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Bacteriostasis of *Escherichia coli* by milk

II. Effect of bicarbonate and transferrin on the activity of infant feeds

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SUMMARY

Fresh human and bovine milk are bacteriostatic *in vitro* for only some (milk-sensitive) strains of *E. coli*. The addition of bicarbonate to the test system potentiates the bacteriostasis so that otherwise milk-resistant strains are inhibited. By titration of the bicarbonate in the milk, it is possible to determine the minimum concentration that will activate milk against a milk-resistant strain but be ineffective in boiled milk, i.e. it potentiates a heat-labile system in milk and does not merely exert a direct toxic effect. This concentration is lower for human milk than for cows' milk and can be reduced even further by the addition of more iron-binding protein.

Lactoferrin and bicarbonate may be present in the gut of the newborn. In an attempt to imitate conditions in the infant gut, we therefore reinvestigated, *in vitro* and in the presence of added bicarbonate and transferrin, the bacteriostatic activity against *E. coli* of fresh breast-milk, commercial bottle-milk, and mixtures of these as fed to infants in this study. The results, and information about events *in vivo* deduced from the ratio of milk-sensitive to milk-resistant strains of *E. coli* isolated from babies' stools, suggest that neonatal intestinal secretions may contribute to the bacteriostatic activity of their feeds so that (1) in fully breast-fed babies all strains of *E. coli* are inhibited to the same extent; there is no selection on the basis of milk sensitivity and equal numbers of strains resistant and sensitive to milk are found in the stools; (2) in fully bottle-fed babies *E. coli* is not inhibited since the milk is non-bacteriostatic and again there is no selection; (3) in babies fed at the breast but bottle-milk supplemented, only milk-sensitive strains are inhibited; milk-resistant strains are not, and preferentially colonize the large intestines.

INTRODUCTION

In the preceding paper, we described the effect of breast feeding on the establishment of *Escherichia coli* in the intestines of new-born infants examined 5 days after birth (Dolby, Honour & Valman, 1977). Strains that were sensitive to the bacteriostatic activity of human milk and strains that were resistant were found in about equal numbers in the stools of bottle-fed infants but babies fed mostly at the breast were colonized with milk-resistant *E. coli*, suggesting that human milk was as effective *in vivo* as well as *in vitro* in suppressing the milk-sensitive strains and allowing only the resistant ones to multiply. We attempted to use

these results to support the suggestion that the bacteriostatic activity of milk plays some part in the protection of the newborn against *E. coli* enteritis (Bullen, Rogers & Leigh, 1972) but two puzzling findings (Dolby *et al.* 1977) had to be explained.

The first was that differences in milk-sensitivity of *E. coli* were independent of serotype. An antibacterial mechanism capable of eliminating only some potential pathogens is not a likely candidate for responsibility for protection *in vivo*.

The second finding was that, of the babies being fed at the breast, only those receiving supplementary bottle feeds of commercially prepared milk were colonized more readily with resistant than with sensitive strains; i.e. in only the 'topped-up' babies did the antibacterial activity of milk appear to be effective *in vivo*. In babies fed completely at the breast, equal numbers of resistant and sensitive strains were isolated just as in wholly bottle-fed babies.

Our milk-sensitivity determinations were done without added bicarbonate whereas Bullen *et al.* (1972) measured the in-vitro antibacterial activity of human milk, which they ascribed to antibody and lactoferrin, in the presence of 0·2% bicarbonate. The potentiating effect of bicarbonate in reactions involving lactoferrin – the iron-binding protein of human milk – is similar (Masson & Heremans, 1968) to that of transferrin (Aisen, Aasa, Malmström & Vänngård, 1967) – the iron-binding protein of serum and of the milk of some animals (Masson & Heremans, 1971). This potentiation is particularly dramatic in cows' milk (Reiter, Brock & Steel, 1975).

We now report an investigation on the effect of bicarbonate and extra iron-binding protein on the antibacterial property of milk and particularly the milk feeds given to infants in our study.

METHODS

Milk

This was from four sources, (*a*) human, expressed breast milk, frozen immediately at −28° C, (*b*) SMA, a processed, 'humanized', bovine milk preparation, (*c*) bovine milk, refrigerated within 4 h and frozen for storage within 24 h, and (*d*) a dried skimmed bovine-milk preparation without alteration or additives. The first two were as used for infant feeding, but the last two were experimental milks for comparison with the first in laboratory tests only.

For use, human milk was thawed and 2–10 ml amounts were heated at 56° C for 30 min ('Milk 56°'); this treatment did not affect its antibacterial activity. Milk heated in a boiling water-bath for 5 min ('Milk 100°') was inactive. Heated milk was stored at 4 °C; V1.76 was a pool of six 5- to 7-day post-partum specimens, V11.75 was a pool of six 3- to 9-week post-partum specimens, and V43 was milk from one mother 5 days *post partum*. Bottle-milk for infant feeds was SMA Gold Cap S26 (John Wyeth and Brother Ltd, Taplow, Maidenhead, Berkshire).

Bovine milk was collected from pooled, pre-pasteurization, routine milkings from two local herds and was stored and heated as the human milk. Dried bovine milk without supplements or 'humanization', for comparison with SMA, was dried skimmed milk (J. Sainsbury Ltd, Stamford Street, London SE1).

Table 1. *Effect of adding sodium bicarbonate to human and bovine milk on the growth of a milk-resistant strain VB*11.2

	No. of times inoculum increased in 3 h at 37° in				
	Human milk pool		Bovine milk pool		
Addition to milk	56° (i)	100° (ii)	56° (iii)	100° (iv)	1% peptone water (v)
None	30	97	31	125	200
Bic 0·2%	0	2	3	75	7
Bic 0·04%	5	70	60	75	100

BIC = Sodium bicarbonate.

The bacteriostatic test

This was performed as before (Dolby *et al.* 1977) except that the pH of the milk was not adjusted; 0·02 ml of a solution of transferrin in phosphate buffer was added to 0·08 ml of milk to give a final concentration of 2 mg/ml. Bicarbonate was supplied as suitable aqueous solutions of $NaHCO_3$ (Analar grade) or by the CO_2 releaser Tris-(hydroxymethyl)-methylamine, Analar grade ('Tris buffer', Fisons, Loughborough) in 0·02 ml.

Bacterial strains

E. coli strains VB11.2 and VB71.1 were milk-resistant strains from the stools of two breast-fed bottle-supplemented babies; V21.1 was a milk-sensitive strain from a mother; strain 0128 was isolated from a child with gastroenteritis by the Department of Microbiology, Northwick Park Hospital and typed by Dr B. Rowe as 0128. K67.H12.

RESULTS AND DISCUSSION

The effect of bicarbonate and of iron-binding protein on bacteriostasis in vitro

Table 1 (line 1) shows the resistance of *E. coli* strain no. VB11.2 to the bacteriostatic action of human and bovine milk; although it did not multiply as fast as in milk 100° or in peptone water the growth in milk 56 °C was still appreciable. The human and the bovine milks were active against the milk-sensitive strain V21.1. The addition of 0·2% sodium bicarbonate (line 2) allowed inhibition of *E. coli* in all but boiled bovine milk but because this concentration of sodium bicarbonate was inhibitory in peptone water, the inhibition in milk was presumably a direct toxic action having nothing to do with the components of milk. Why was it non-toxic in boiled bovine milk?

Reiter *et al.* (1975) showed that citrate ions prevented the bicarbonate potentiation of the bacteriostatic activity of milk. Only when bicarbonate ions were in excess of citrate did bacteriostasis occur. Bovine milk contains 2–8 times as much citrate as human milk (Jenness, 1974) so that the effective bicarbonate concentration of bovine milk is much less than this and, as shown in Table 1 (line 2, column

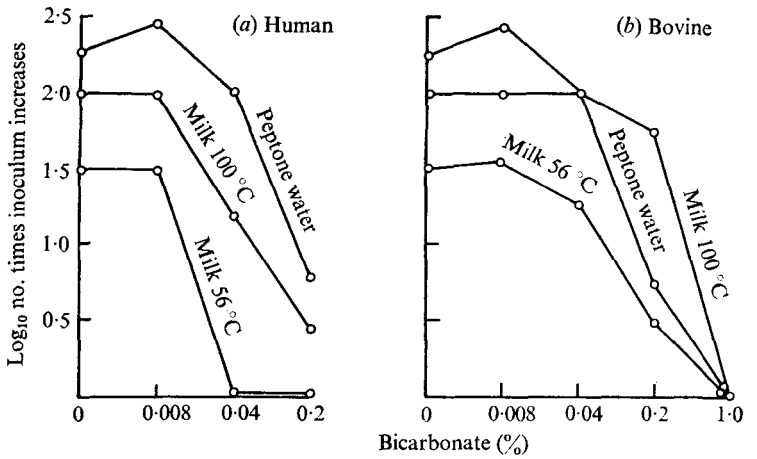

Fig. 1. The bacteriostasis of milk-resistant strain VB11.2 by human milk V11.75 (*a*) and by bovine milk (*b*) at increasing concentrations of bicarbonate in milk heated to 56° and 100° and peptone water.

Table 2. *The effect of the addition of transferrin and sub-optimal concentrations of bicarbonate to human milk on the growth of a milk-resistant strain VB*11.2

Growth of *E. coli* in	No. of times inoculum increased in 3 h at 37°: Milk V43, strain VB11.2	Milk pool V1.76, strain 0128
Milk 56°	45	85
+Bic*	25	72
+TF†	36	70
+Bic/TF	6	12
Milk 100°	190	195
+Bic	50	120
+TF	50	100
+Bic/TF	55	100

* Bic: 0·04% sodium bicarbonate.
† TF: 2 mg per ml transferrin.

(iv)), non-toxic. The inhibition caused by this concentration in unboiled bovine milk was therefore a potentiation of the heat-labile bacteriostatic systems and not a direct toxic action of bicarbonate (line 2, column (iii)). A concentration of 0·04% sodium bicarbonate achieved a similar effect in human milk (Table 1, line (3)).

The concentrations of bicarbonate required to potentiate milk bacteriostasis against an otherwise resistant *E. coli* are shown for human and bovine milk in Fig. 1. The 'titration' was done for each milk on 56° and 100° heated pools, and confirmed that the effective average concentrations were about 0·04% bicarbonate for human milk and 0·2% for bovine milk. Although not shown in Fig. 1, 0·07% Tris – the bicarbonate-ion releaser – was about as effective as 0·04% $NaHCO_3$.

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We next found that the addition of transferrin to concentrations of bicarbonate in milk 56° which were just below those capable of potentiating bacteriostasis against the resistant strain made the milk active. Transferrin added in the absence of bicarbonate was without effect. This is shown in Table 2 for a single milk specimen against *E. coli* VB11.2 and for pooled human milk against a strain of enteropathogenic serotype. Transferrin at 2 mg/ml was added in all our experiments but one-tenth this amount was found to be effective on the two occasions tested.

From the experiments we have described, it seems that milk alone is active against some strains of *E. coli*, which we have called milk-sensitive strains. In the presence of bicarbonate, it is active against all strains; this effect is achieved by lower concentrations of bicarbonate if more iron-binding protein is added. If the infant can supply bicarbonate and iron-binding protein, bacteriostasis by milk might be a mechanism active against all *E. coli* and therefore responsible for protection *in vivo* against all pathogenic *E. coli*, and might be one of the explanations for the protection conferred by suckling.

In the adult pancreatic bicarbonate is present at 30 mmol/l or about 0·2%. As we have seen, this concentration has a non-specific toxic effect against *E. coli* even in boiled milk. Because boiled milk is unable to protect the newborn from *E. coli* enteritis (Tassovatz & Kotsitch, 1961), either the newborn does not produce this amount of bicarbonate or this toxic effect is an in-vitro artefact. The bicarbonate secretion of 2-month-old babies investigated by Delachaume-Salem & Sarles (1970) and of one baby at 1 month (Gibbs, 1950) was as high as in adults. It is likely therefore that in younger children some bicarbonate is secreted.

We used serum transferrin for increasing the concentration of iron-binding protein and thus decreasing the bicarbonate requirement for the potentiation of the activity of milk against all strains. Lactoferrin, found in many secretions, is more likely to be present in the gut. Although Tourville, Adler, Bienstock & Tomasi (1969) were unable to demonstrate it in adult intestines, its presence in the stomach and whole length of the adult gut was reported by Masson, Heremans, Schonne & Crabbe (1969), who found concentrations of about 50 μg/ml in gastric juice but much less in duodenal juice. Transferrin can be produced by fetal tissues as early as at 8 weeks (Gitlin & Biasucci, 1969) and is present in the serum of a full-term baby at concentrations approaching that in the adult (Toivanen, Rossi & Hirvonen, 1969). Perhaps it is not unreasonable to suppose that lactoferrin might also be secreted from birth.

From the above, it seems that a newborn baby might be able to produce at least low concentrations of bicarbonate and some lactoferrin. We therefore determined the antibacterial activity *in vitro* of milk and milk mixtures, as fed to infants in our previous study, in the presence of bicarbonate and transferrin to find out whether these additions to the test produced conditions that imitated those *in vivo* as illustrated by the strains of *E. coli* excreted.

Table 3. *Bacteriostatic activity of human milk and SMA with and without added sodium bicarbonate and transferrin on milk-sensitive and milk-resistant strains of* E. coli

		Number of times inoculum increases in				
				Hu 56°/SMA mixtures		
Additives	*E. coli*	Hu 56°	SMA	3:1	1:1	1:3
None	S	2	55	8	60	60
	R	60	100	120	100	110
Bic 0·04%/TF	S	1	50	4	7	24
	R	8	100	120	160	150
Bic 0·2%/TF	R	—*	63	41	65	30

* Not relevant because added bicarbonate was at toxic level. Human milk is shown as Hu, sodium bicarbonate as Bic, and transferrin 2 mg/ml as TF. The strains of *E. coli* were either milk-sensitive (S), V21.1 or milk-resistant (R) VB11.2 or VB71.1.

Table 4. *Association of the type of feed on selection of* E. coli, *expected and found, in the intestines of neonates*

	In-vitro bacteriostasis		In-vivo colonization	
Type of feed	Sensitive	Resistant	Expected	Found
Human milk	+	+	Equal nos.	Both
SMA	−	−	Equal nos.	Both
Human milk SMA supplemented	+	−	More resistant	Resistant

The in-vitro activity of milk feeds and the colonization of infants' intestines by milk-resistant and milk-sensitive E. coli

Table 3 shows the growth of a milk-sensitive and a milk-resistant strain of *E. coli* in infant feeds and in those feeds with sodium bicarbonate, 0·04% and transferrin 2 mg/ml such as might be present in the infant's small intestine. Without additions the milk from the breast alone or with dilution by SMA of not more than 25% was active only against the sensitive strain. In the presence of bicarbonate 0·04% and transferrin 2 mg/ml although breast milk alone was now active against both strains none of the milk/SMA mixtures was active against the resistant strain. Dilution of breast milk with up to 50% SMA, however, did not reverse the inhibition of breast milk against the sensitive strain. Because of the extra citrate contributed by the cows'-milk-based SMA the bicarbonate could be increased up to 0·2% without becoming toxic but bacteriostasis of a resistant strain was still absent.

Table 4 summarizes these results in terms of bacteriostasis and resultant selection of organisms by these mixtures; the 'expected' and 'found' results agree. This suggests that by choosing conditions to achieve this, our in-vitro tests are imitating events in the gut of a breast-fed, a bottle-fed, or a breast-fed bottle-supplemented baby. If one assumes therefore that the bacteriostasis is

potentiated by bicarbonate and possibly augmented by more iron-binding protein, the colonization of the baby by *E. coli* as actually found and described previously (Dolby *et al.* 1977) can now be explained. The unexpected finding of lack of selection in completely breast-fed babies can now be seen as the result of equal bacteriostasis of all strains in the gut; this mechanism could therefore contribute towards protection against *E. coli* multiplication in the small bowel.

The effect of SMA 'topping up' is really the effect of diluting human milk in SMA and, as we have seen, causes a loss of the bacteriostatic power of human milk against all strains even in the presence of bicarbonate and transferrin. To determine whether SMA counteracted the bacteriostasis merely by dilution or because it was bovine milk with a high citrate concentration, because it was supplemented with iron (Department of Health and Social Security, 1974) or because of its preparation, we compared the effect on bacteriostasis of diluting human milk 56° with (i) cows' milk 100°, (ii) human milk 100°, (iii) skimmed milk dried and reconstituted. These experiments showed that the reduction of bacteriostasis that occurs when human milk is mixed with SMA is partly due to its being made from bovine milk and partly due to the added iron. Dilution of human milk 56° in human milk 100° has a much less adverse effect on bacteriostasis because citrate and iron concentrations are the same; equal parts of fresh or 56° milk and 100° milk for example are still moderately bacteriostatic.

In a recent investigation Gothefors *et al.* (1976) have shown that in breast-fed babies antibody against the somatic O-antigen of *E. coli* milk and cord serum are of no importance in the control of gut colonization and of perhaps enteritis. They ask what other mechanism could be involved; we suggest that the O-antibody independent bacteriostatic properties of milk may be able to control *E. coli* in the infant gut by some yet unknown mechanism.

If, as seems likely from the above, bicarbonate ions are involved in antibacterial mechanism at mucosal surfaces, low bicarbonate in secretions in children with cystic fibrosis (Hadorn *et al.* 1968) might account for the frequency of infection in these children, particularly with Enterobacteriaceae (Seidom, Mosovitch & Neter, 1975).

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